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ABSTRACT

The ITS DNA marker is routinely used for fungal identification but gives a clear result for only three out of four powdery mildew samples. A search for new markers indicates that some genes offer enhanced identification in comparison with ITS. Others fail due to amplification and sequencing difficulties and lack of informative variability. Powdery mildews (Ascomycota, Erysiphales) are biotrophic, fungal plant pathogens, which commonly occur worldwide on a wide range of host plants. They are unsightly and greatly reduce the vigour of their hosts and have major impacts on crops and other cultivated plants. Species within this order are straightforward to spot, but difficult to identify. A citizen science scheme was run 2013-2016 in the UK to gather a wide array of samples on which identification methods could be developed. Current techniques for identification and phylogenetic reconstruction show scope for improvement. In this paper we review genes used in other fungal groups for discrimination at species level. Working protocols for amplification and sequencing of seven genes (Actin, β-tubulin, Calmodulin, *Chs*, EF1-α, *Mcm7*, and *Tsr1*) are developed with varying success; *Mcm7* proves to be the most useful at differentiation between closely related, phylogenetically young powdery mildew species for phylogenetic reconstruction when used separately and in tandem with ITS. We therefore propose this as the most appropriate candidate gene to be used commonly in powdery mildew diagnostics alongside the ITS, furthermore this could be transferred to similarly troublesome fungal clades.

KEY WORDS Erysiphales, powdery mildew, citizen science, survey, BLAST, phylogeny, molecular markers, ITS, Mcm7.

INTRODUCTION

Powdery mildews (PMs) are some of the most diverse and frequently encountered plant pathogenic fungi in the world (Braun, 1987). The 872 species described by Braun and Cook (2012) form a characteristic white talcum powder-like coating on the surfaces of approximately 10,000 different host plants (Braun, 1987) including economically important crops and ornamentals. Accurate identification is essential for effective control; however these obligate biotrophs pose problems for taxonomists as many species lack clear morphological characters to distinguish them, are difficult to culture, and therefore require molecular techniques to discriminate between them. Traditional identification methods depend upon a combination of host plant identity and fungal morphology, although more recently sequencing of genomic rDNA regions have come to be used. The internal transcribed spacer (ITS) (Schoch et al., 2012) was proposed as the anchoring barcoding region for fungal identification and has been used extensively within the Erysiphales (Kovács et al., 2011; Wang et al., 2013). Such studies have furthered the efficiency and accuracy of PM species identification, but leave scope for improvement; discrepancies remain in the consistent separation using phylogenetic and barcoding analyses of closely-related, phylogenetically young species.

Fungal phylogenomics has led to an increase in the use of low-copy number protein-coding genes for resolving deep or species-level phylogenies (Aguileta et al., 2008; Schmitt et al., 2009; Curto et al., 2012). These are useful at species level because of their sometimes rapid evolutionary rate (Sang, 2002; Small et al., 2004; Choi et al., 2006). However, given the high evolutionary rate inherent in some low-copy regions, markers may not work consistently among sister lineages because the primer sites may differ. In cases of rapid speciation, DNA may not have diverged sufficiently to resolve a phylogeny using a single locus (Beltrán et al., 2002; Seehausen et al., 2003). This has been shown to be the case in numerous clades (Reeb et al., 2004; Raja et al., 2011; Morgenstern et al., 2012), including the PMs (Khodaparast et al., 2001; Heluta et al., 2010; Kabaktepe et al., 2017). However, multiple independent loci can often provide the necessary variability for reliable species

identification using phylogenetic analyses and DNA barcoding (Beltrán et al., 2002; Sang, 2002) due to the greater level of sampling of the genome. The process of finding markers that are effective at generating species-level discrimination are essential for developing molecular approaches to identification.

Potentially useful, understudied regions, can be mined from four published genomes; *Blumeria graminis* (Spanu et al., 2010; Wicker et al., 2013), *Erysiphe necator* (Jones et al., 2014), and *Erysiphe pisi* and *Golovinomyces orontii* (Max Planck Institute for Plant Breeding Research). Through analyses of alignments of the PM genomes alongside closely-related Ascomycota, primers can be developed for exclusive amplification of PMs.

This data mining has been largely driven by phylogenetic reconstruction efforts, initially at familial and generic levels, but now focused on species (Inuma et al., 2007; Takamatsu et al., 2008a; Takamatsu et al., 2008b; Takamatsu, 2013; Meeboon et al., 2015; Meeboon & Takamatsu, 2015a, b, c). The use of additional DNA regions in tandem with ITS can increase phylogenetic resolution and stability at multiple taxonomic levels. A standard, broadly applicable, PM specific, set of sequence markers would be a valuable resource in constructing robust PM phylogenies using only a few loci (Schmitt et al., 2009) without the expense of whole genome sequencing, while also informing studies of other fungi. A further problem is the risk of contamination from host or hyperparasite DNA, which is normally overcome by growing the required Fungi in single spore culture, however, PM culturing trials have rarely succeeded (Morrison, 1960; Kenyon et al., 1995; Álvarez & Torés, 1997; Nicot et al., 2002).

Numerous regions have been used routinely to identify species and infer evolutionary relationships within the Ascomycota. A review of literature highlighted genes worthy of detailed investigation in the PMs. This shortlist includes: Actin (McElroy et al., 1990; Reece et al., 1992; Baldauf et al., 2000; Voigt & Wöstemeyer, 2000; Daniel et al., 2001; Voigt & Wöstemeyer, 2001; Daniel & Meyer, 2003; Yun et al., 2003; Opalski et al., 2005; Hunter et al., 2006), ß-tubulin (O' Donnell et al., 1998b; de Jong

et al., 2001; McKean et al., 2001; Einax & Voigt, 2003; Juuti et al., 2005), Calmodulin (Stevens, 1983; O' Donnell et al., 2000; Mulè et al., 2004; Wang & Zhuang, 2007; Madrid et al., 2009; Romeo et al., 2011; Samson et al., 2014), Chitin synthase (*Chs*) (Roberts et al., 1986; Kano et al., 1997; Debono & Gordee, 1994; Zhang et al., 2000; Kong et al., 2012), Elongation factor 1 alpha (EF1-a) (O' Donnell et al., 1998a; Roger et al., 1999; Baldauf et al., 2000; Seifert & Lévesque, 2004; Kristensen et al., 2005; Hunter et al., 2006; Maphosa et al., 2006; Matheny et al., 2007; Amatulli et al., 2010), *Mcm7* (Moir et al., 1982; Kearsey & Labib, 1998; Aguileta et al., 2008; Schmitt et al., 2009; Leavitt et al., 2011; Raja et al., 2011; Divakar et al., 2012; Morgenstern et al., 2012; Minnis & Lindner, 2013; Tretter et al., 2013; Tretter et al., 2014; Prieto & Wedin, 2016), and *Tsr1* (Gelperin et al., 2001; Schmitt et al., 2009; Tretter et al., 2013; Sadowska-Deś et al., 2013). In the present study the possibility of developing working molecular markers for these regions for PM samples collected through a citizen science survey in the UK was investigated. The value of resultant data for species identification using phylogenetic reconstruction is explored with reference to the ITS region.

MATERIALS AND METHODS

Sample collection – The Powdery Mildew Survey—A total of 596 samples were collected from around the UK via the powdery mildew citizen science scheme (article in preparation). Contributors were asked to submit PM samples via the postal service along with GPS data. Any supplied host identifications were checked after receipt and all PM identifications were carried out by the researchers. Fungal packets and extracted DNA were deposited in Reading Herbarium (RNG). Five hundred and seven of these were successfully amplified using previously designed ITS markers PMITS1 and PMITS2 (Cunnington et al., 2003). These acted as reference sequences for later sequencing of novel DNA regions.

Initial species identification.—Putative species identification was achieved using previously established methods: host plants were identified using the Vegetative key to the British Flora (Poland & Clement, 2009) and confirmed by comparison with standard herbarium specimens at

RNG; fungi were mounted on slides and imaged using a Leica DM2000 LED with associated Leica Application Suite, and morphological features noted according to Braun and Cook (2012); and rDNA ITS sequence data were analysed using Megablast (Altschul et al., 1990) with a threshold of 99% similarity indicating allegiance to specific PM species (Tang et al., 2017).

DNA isolation.—DNA was extracted from 0.01-0.02g dry weight of infected leaf material. Leaf material was frozen using liquid nitrogen and ground with two tungsten carbide ball bearings and acid washed silica sand using the Qiagen TissueLyser II. The Qiagen DNeasy Plant Mini Kit protocol was then followed without modification.

Data mining for molecular markers and DNA amplification.—Generic primers listed in SUPPLEMENTARY TABLE 1 were initially trialled with limited success for amplifying PM amplicons.

PM genomes (Spanu et al., 2010; Wicker et al., 2013; Jones et al., 2014) were aligned with closely related ascomycetous species of the genes in question (TABLE 1), using MUSCLE (Edgar, 2004) and manually edited such that all bases were in the correct amino acid reading frame, in order to identify the location of the candidate genes and then develop primers to trial in the amplification of accessions. Primers were designed manually with the aid of the web-based software Primer-BLAST (Ye et al., 2012) and Primer3 (Untergasser et al., 2012). The optimal primer-choice conditions were set for a length of approximately 20 bases, annealing temperature (Tm) of 60 °C, and GC content of around 50%. Default salt concentrations were used in each case. Primer pairs were developed to produce amplicons of 300-1300 bp (depending upon gene) (SUPPLEMENTARY TABLE 2) and ordered from Sigma-Aldrich. Primers shown to have the greatest amplification and sequencing success and therefore deemed the most useful are listed in TABLE 2.

PCR was carried out using the newly designed primers for five individual gene regions. All possible primer combinations resulting in amplicons of greater than 200 bp were trialled; the most successful (success = number of products x product strength) were further trialled at a gradient of annealing

temperatures until a single combination was finalised for each candidate gene. 25 µl PCR mixes comprised of 12.5 µl BioMix™ Red (Bioline), 0.75 µl BSA (10 ng μl^{-1}), 0.875 µl of each primer at 10 µM, 9 µl RO water, and 1 µl of sample DNA at concentrations of 10-50 ng μl^{-1}. Cycling parameters were adapted from Amrani and Corio-Costet (2006) with an initial denaturation step of 95 °C for five minutes, followed by 37 cycles of denaturation at 95 °C for 30 seconds, annealing for one minute at varying temperatures for different genes, and elongation at 72 °C for one and a half minutes and a final elongation at 72 °C for five minutes. Single amplicons of more than 10 ng per band were then sent to Source BioScience in 2014 and 2015, and GATC in Germany in 2016 for sequencing. Complementary forward and reverse sequences generated in this study were assembled and manually edited using SeqMan Pro software (DNASTAR, Madison, WI, USA) and deposited in GenBank. Reducing the degeneracy and length of the most successful primers was trialled for greater accuracy in amplification and sequencing but rarely improved success.

Phylogenetic analyses.— Alignments of resultant sequence data were performed using MUSCLE (Edgar, 2004) before a manual edit. Accessions within alignments of ITS, Actin, β-tubulin, *Mcm7*, and *Tsr1* were reduced and trimmed as a result of poor sequence quality and short reads and were concatenated in 26 possible combinations using MESQUITE (Maddison & Maddison, 2017). Alignment and tree files were deposited in TREEBASE (TABLE 3).

The optimal nucleotide substitution model was selected for each alignment based on the AIC criterion (Akaike, 1974) using PAUP (Swofford, 2003) and MRMODELBLOCK commands generated by MRMODELTEST (Nylander, 2004). Bayesian inference (BI) was performed in MRBAYES 3.1.2 (Ronquist & Huelsenbeck, 2003). Combined analyses were run using partitioned models and therefore included optimal nucleotide substitution models for each individual gene alignment. Stationarity was established using a plot of –ln, a 25% burn-in was used and all trees were rooted with *Blumeria graminis* (“5_86_Blumeria_graminis_ex_Poa_trivialis”) in order to make topologies comparable. All other variables followed default settings of MrBayes. Parameter states and trees were stored every

10,000 generations to avoid autocorrelation. To ensure convergence was reached, the average standard deviation of split frequencies was monitored to ensure that it fell below 0.03, and trace files of all parameters were examined using Tracer v1.6 (Rambaut et al., 2015) to ensure proper mixing had happened. Consensus of resultant trees was built and visualised using BAYESTREES 1.3 (http://www.evolution.reading.ac.uk/BayesTrees.html).

Phylogenies inferred for each combination of regions were compared with the total evidence tree (TET) and relative consensus fork indexes (RCFI) were estimated to give the proportion of nodes shared among the trees (Colless, 1980) (TABLE 3).

RESULTS

Candidate regions.—

Actin. ActF1 and ActR3 showed the greatest success in amplification and sequencing. Degeneracy in these primers was reduced based on initial sequence data, and primers ActF1a and ActR3b were developed and used in all future amplifications to amplify a product of 500 bp with an annealing temperature of 60 °C. Sequences were contributed to GenBank (Accession numbers KY786551 – KY786689).

β-tubulin. BTF5 and BTR7 showed the greatest success in amplification and sequencing. Degeneracy in these primers was reduced based on initial sequence data and primers BTF5b and BTR7a were developed. This was used in all future amplifications to amplify a product of 800 bp with an annealing temperature of 55 °C. Sequences were contributed to GenBank (Accession numbers KY786690 – KY786781).

Calmodulin. The Calmodulin region did not show sufficient variability from closely related ascomycete fungi for development of PM specific primers.

Chitin synthase. The *Chs* region did not show sufficient variability from closely related ascomycete fungi for development of PM specific primers.

EF1-α. All primer combinations resulted in amplification of multiple products. Extension and annealing temperatures and concentrations of primer, DNA and magnesium were therefore explored. However, no combination was shown to consistently produce a single product and the region was explored no further.

Mcm7. Primer combination Mcm7F2 and Mcm7R8 initially showed the greatest success in amplification and sequencing. Degeneracy in these primers was reduced based on initial sequence data and primers but application of new markers produced sequences of lesser quality. Mcm7F2 and Mcm7R8 were therefore used in all future amplifications to amplify a product of 550 bp with an annealing temperature of 54 °C. Sequences were contributed to GenBank (Accession numbers KY786340 – KY786476).

Tsr1. Primer combination Tsr1F1 and Tsr1R6 initially showed the greatest success in amplification and sequencing. Degeneracy in these primers was reduced based on initial sequence data and primers but application of new markers produced sequences of lesser quality. Tsr1F1 and Tsr1R6 were therefore used in all future amplifications to amplify a product of 1150 bp with an annealing temperature of 52 °C. Sequences were contributed to GenBank (Accession numbers KY786477 – KY786550).

Species identification and sample selection, amplification and sequencing.— Morphological examinations of 596 samples enabled identification to PM genus 80% of the time and to PM species 65% of the time. Various subsets of all 596 accessions were successfully amplified and sequenced for each candidate gene (TABLE 4). As expected, NCBI BLAST of ITS sequences enabled identification to genera, but less often to species (TABLE 5). For candidate genes this resource was of little use due to the previous lack of existing sequence data (TABLE 5).

Phylogenetic analyses.— Phylogenies constructed from individual regions enabled evaluation of the relative accuracy of each region when compared to the most commonly used region, the ITS (FIG 1), as well as an ideal total evidence tree. RCFIs displayed in TABLE 3 enable comparison and highlight the sub-optimal Actin gene (RCFI = 0.684), the β-tubulin (RCFI = 0.874) and *Tsr1* (RCFI = 0.857) genes performing similarly to the ITS (RCFI = 0.879), and the *Mcm7* gene reconstructing a close to optimal tree (RCFI = 0.974). The most informative phylogenies are included (FIG 2, FIG 3, and FIG 4).

The 495 bp alignment of 151 *Mcm7* PM accessions (FIG 3) had strong support across all clades, however, relationships within Golovinomyceteae show *Neoerysiphe* to be sister to *Erysiphe*, therefore indicating that Golovinomyceteae might be a paraphyletic tribe as currently defined.

The 1315 bp alignment of 102 accessions of the ITS and *Mcm7* regions combined has RCFI of 0.972 and high PP support for each tribe and genus (FIG 4). RCFI reaches 1 when combining the four regions of ITS, *Mcm7*, β-tubulin, and *Tsr1* and ITS, *Mcm7*, β-tubulin, and Actin as well as when all five regions were combined (TABLE 3).

DISCUSSION

Morphological analyses of PM species (Braun & Cook, 2012) enable correct identification much of the time but require specialist knowledge, access to a high quality microscope, and visual discrimination between microstructures. Complementing morphology with sequencing of rDNA ITS (White et al., 1990; Takamatsu et al., 1998; Tang et al., 2017) has led to improvements in accuracy and efficiency of species identification in the PMs, as well as other Fungi. The abundance, diversity, and quality of PM accessions and ITS sequence data in the current study proved sufficient for testing established PM identification techniques; enabling a high proportion (c. 80%) of species to be discriminated. However, many accessions such as those on *Heuchera* cultivars (Ellingham et al., 2016) yielded ambiguous results using NCBI GenBank BLAST, either due to a lack of relevant ITS data or a lack of variability in deposited ITS data, as well as the presence of misidentified accessions

(Kovács et al., 2011). ITS has regularly proven to be insufficient for PM species discrimination (Meeboon et al., 2015; Takamatsu et al., 2015; Fonseca et al., 2017) and alternative housekeeping genes have proven to be of varying use for solving this issue (Vela-Corcía et al., 2014; Pirondi et al., 2015).

This was also the case for gene regions tested in the current study to assess their efficacy for species recognition: Calmodulin and *Chs* alignments lacking priming sites for exclusive amplification of PMs from environmental samples; developed EF1-α primers lacking specificity to PMs; Actin sequences proving to be too variable; only the β-tubulin, *Mcm7*, and *Tsr1* genes showing promise. Amplification, sequencing, and species discrimination success varied greatly across genes, perhaps due to low quantity and quality of environmental PM DNA. Those which were unsuccessful were characterised by weak reads, resulting in little or no sequence data, or messy reads, potentially contaminated with more than one PM species or with mycoparasitic fungi (Pintye et al., 2015; Topalidou & Shaw, 2016) and other phylloplane community members (Berendsen et al., 2012; Topalidou, 2014; Franco et al., 2017). Amplification of multiple products in certain accessions was also evident and may indicate that the primer combination is not 100% specific to PMs; amplifying mycoparasitic fungi as well as the targeted PM. It is also possible that more than one PM species was present in the accession as in certain cases DNA sequences of different genes returned disparate species identifications. These were taken particularly seriously in cases of disparity between *Mcm7* and ITS because they may signal the presence of more than one PM species on the host plant (Cook et al., 2006).

Success in amplification and sequencing of specific PM genes enabled analytical techniques to be evaluated. GenBank MegaBLAST was of little use owing to the dearth of sequence data. This is unsurprising due to the novelty of the genes investigated. However, BLAST within the local dataset (those generated in the current study) proved intraspecific accessions to return highly similar matches within the β-tubulin, *Mcm7*, and *Tsr1* regions.

Bayesian analyses of the datasets in TABLE 3 resolved numerous monophyletic groupings of distinct taxa. However, while analyses based on β-tubulin, as well as *Tsr1*, did produce phylogenies of similar resolution to that of ITS, *Mcm7* proved superior to each: resolving 97% of all accessions. The utility of each novel gene has varied across previous studies. The Actin gene in particular has received both positive (Baldauf et al., 2000; Voigt & Wöstemeyer, 2000; Daniel et al., 2001; Voigt & Wöstemeyer, 2001; Daniel & Meyer, 2003) and negative (Weiland & Sundsbak, 2000; Hunter et al., 2006) reviews. In contrast, the few studies to have used *Tsr1* have been mostly positive (Schmitt et al., 2009; Sadowska-Deś et al., 2013; Tretter et al., 2013), however, as in the present study, it was not considered to offer sufficient resolution in the study of Morgenstern et al. (2012). β-tubulin has regularly emerged as a strong candidate for complementing the ITS in fungal clades including but not limited to *Neofabraea* species causing tree cankers and bull's eye rot of apple (de Jong et al., 2001), and the *Gibberella fujikuroi* (*Fusarium*) species complex (O' Donnell et al., 1998b). Its utility has also been shown in population genetics studies of PMs (Cunnington et al., 2003; Inuma et al., 2007; Brewer & Milgroom, 2010; Troch et al., 2014; Vela-Corcía et al., 2014). These concur with the current study. *Mcm7* proved consistently to enable a high-level of resolution across various fungal clades (Aguileta et al., 2008; Schmitt et al., 2009; Leavitt et al., 2011; Raja et al., 2011; Divakar et al., 2012; Morgenstern et al., 2012; Tretter et al., 2013; Tretter et al., 2014; Prieto & Wedin, 2016).

Phylogenetic analyses of candidate genes have shown the utility of each; and proved the great potential of *Mcm7* for improving PM diagnostics. The region should be adopted to aid future identification of PM species. Improved efficacy and accuracy of diagnostically useful molecular markers of plant pathogens could be paired with new technologies such as lateral flow microarrays (Carter & Cary, 2007) or direct PCR (Werle et al., 1994) and on-site sequencing using nanopore technologies such as the MinION (Eisenstein, 2012; Mikheyev & Tin, 2014; Mitsuhashi et al., 2017). These could offer fast and efficient assays for disease identification for plant health practitioners. *Mcm7* must now be tested with broader sampling of PMs from undersampled geographic regions

and genera. The candidate *Mcm7* primers could be adapted further to improve amplification for specific genera.

The ITS region has historically underpinned fungal diagnostics and, due to the large amount of ITS sequence data already deposited in online sequence databases, is likely to continue to do so. The β-tubulin region can offer additional diagnostic utility to the PM identification process; serving as an 'identifier' complementing ITS as the 'anchor' region. The *Mcm7* region offers greater promise still and would be an ideal candidate to replace ITS for PMs and potentially all Fungi. However, the existing weight of ITS data means it remains the most widely sequenced identifier of PMs and other fungal clades.

While much work remains, the results obtained in the current study have confirmed: (i) the promise of underexploited markers at complementing existing gene regions in species level discrimination; and (ii) the potential of new techniques to develop rapid diagnostic methods for PMs, and other plant pathogenic fungi.

Consistently rapid and accurate PM identification could benefit researchers and practitioners in this field: (i) to limit the spread of plant pathogenic fungi and minimise the occurrence of devastating disease outbreaks on monoclonal crops and other cultivated plants (Jørgensen, 1992; Frye & Innes, 1998; Liu et al., 1999); (ii) contribute to more effective chemical (Horst et al., 1992; Faoro et al., 2008) and cultural (Xiao et al., 2001) control, influenced by pathogen species or races; (iii) contribute to breeding programmes aimed at developing disease resistant plants; and (iv) determine whether a pathogen is native or introduced.

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LEGENDS

Figure 1: A selection of PM infected hosts collected during the powdery mildew survey: (a) unidentified PM on *Monarda didyma*, (b) *Neoerysiphe galeopsidis* on *Acanthus spinosus*, (c) *Erysiphe pisi* on *Pisum sativum*, (d) *Podosphaera leucotricha* on *Malus domestica*, (e) *E. trifoliorum* on *Trifolium arvense*, (f) *E. aquilegiae* on *Aquilegia vulgaris*, (g) *E. berberidis* on *Berberis thunbergii*, and (h) *E. catalpae* on *Catalpa bignonioides*.

Figure 2: BI using 173 sequences of the ITS region, RCFI = 0.879. Accession names include accession code, PM name, and host identity. PPs ≥90% are shown in blue. Green boxes denote PM tribes.

Figure 3: Part 1 of BI using 151 sequences of the Mcm7 region, RCFI = 0.974. Accession names include sample code, PM name, and host identity. PPs ≥90% are shown in blue. Green boxes denote PM tribes.

Figure 4: BI using 102 sequences of the ITS and Mcm7 regions combined, RCFI = 0.972. Accession names include accession code, PM name, and host identity. PPs ≥90% are shown in blue. Green boxes denote PM tribes.

FOOTNOTE

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